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Thin Layer Chromatographic Analysis of Neutral Lipids and Phospholipids in *Helisoma trivolvis* (Colorado Strain) Maintained on a High Fat Diet

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Abstract. Thin layer chromatographic analysis was used to determine neutral lipids and phospholipids in *Helisoma trivolvis* (Colorado strain) snails maintained on a high fat diet of hen's egg yolk. Whole snail bodies, the digestive gland-gonad complexes (DGGs), and plasma samples were analyzed for lipids. The mean percentage values of free sterols, free fatty acids, triacylglycerols, methyl esters, steryl esters, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) in snail whole bodies fed yolk were 1.9, 2.3, 9.2, 2.8, 63, 3, and 5.6 times higher, respectively, than those fed lettuce. The mean percentage values of free sterols, free fatty acids, triacylglycerols, methyl esters, steryl esters, PC, and PE in the DGGs of snails fed yolk were 3.0, 2.2, 3.0, 6.4, 58, 1.4, and 3.4 times higher, respectively, than those on the lettuce diet. Only free fatty acids and PC were detected in plasma, and the respective concentrations were 1.9 and 3.4 times higher in the plasma of the snails on the yolk diet compared to those on the lettuce diet. This research shows that *H. trivolvis,* in addition to *Biomphalaria glabrata,* is a useful pulmonate to study the effects of a high fat diet on hyperlipidemia and hyperlipemia in snails.

INTRODUCTION

Fried et al. (1989) induced elevated levels of lipids in the tissues and hemolymph of the medically important planorbid snail *Biomphalaria glabrata* (Say, 1816) by feeding snails on a high lipid diet of hen's egg yolk. *Biomphalaria glabrata* maintained on the egg yolk diet have been used to study nutrition in uninfected snails and those infected with larval schistosomes and echinostomes (see reviews in Fried & Sherma, 1990, 1993).

One of us (BF) has maintained a continuous culture of a Colorado strain of the planorbid *Helisoma trivolvis* (Say, 1816) for about 15 years. This strain is refractory to miracidial infection by schistosomes and echinostomes, lacks melanin, has an orange-red body, and has been used extensively in neurobiology (see Kater, 1974). This snail is easy to maintain in the laboratory on a leaf-lettuce diet, and an earlier study by Park et al. (1991) examined neutral lipids in an *H. trivolvis* strain maintained on a lettuce-Tetramin diet. To date the only studies on the effect of high fat diet on snails are those that use *B. glabrata.* The purpose of this study was to show the usefulness of *H. trivolvis* as a model to study the effects of a high lipid diet on hyperlipidemia and hyperlipemia in a pulmonate snail. *H. trivolvis* is easier to maintain in simple cultures at room temperature than is *B. glabrata* and provides a useful model for experimental dietary studies.

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MATERIALS AND METHODS

Stock cultures of *Helisoma trivolvis* (Colorado strain) were maintained from eggs to sexually mature adults at 23 ± 1°C in aerated glass jars each containing 10 to 20 snails in 800 mL of artificial spring water (ASW). The ASW was prepared as described by Ulmer (1970). One culture of 15 snails with shell lengths ranging from 16–20 mm was maintained *ad libitum* on boiled Romaine leaf lettuce (L diet) for 20 weeks. Another culture of 13 snails was maintained on a boiled leaf lettuce diet for 16 weeks and then on a boiled hen's egg yolk diet (Y diet) *ad libitum* for an additional 4 weeks. Food and water were changed three times per week in all cultures.

For TLC analysis, the whole bodies and digestive gland-gonad complexes (DGGs) of four individual snails (n = 4) on each of the Y and L diets were prepared. Hemolymph from three snails was pooled to make a sample containing 300 μL of blood from the L diet (n = 1): likewise, hemolymph was pooled from three snails on the Y diet to prepare a 125 μL sample from that population (n = 1). Shells of all snails were removed by gently crushing with a hammer, and the snail bodies were removed with forceps. The DGGs were dissected free of the visceral mass using forceps, and pooled hemolymph was removed using a Pasteur pipet.

Whole bodies and DGGs were prepared by homogenizing each sample in 1 mL of chloroform-methanol (2:1) in a glass homogenizer. The sample was washed twice with 1 mL of chloroform-methanol (2:1), and the supernatant was passed through a glass wool filter and treated with Folch wash (0.88% KCl, 1 mL). Pooled hemolymph

was centrifuged for 6 min at 8000 rpm, and the plasma was removed and treated with 2 mL of chloroform-methanol (2:1), and then passed through a glass wool filter. The filtrate was treated with 1 mL of Folch wash. All samples were placed in a refrigerator at 4°C for 1 hr to separate the biphasic layer. The upper hydrophilic layer was discarded using a Pasteur pipet, and the lipophilic layer was transferred to a 7 mL glass vial and dried under nitrogen gas in a water bath at 40°C. Plasma samples of the L and Y diets were each reconstituted with 75 μL of chloroform-methanol (2:1). DGG and whole body samples of the L diet were reconstituted with 200 μL of chloroform-methanol (2:1) and the Y diet samples were reconstituted with 400 μL of this solvent.

The standard for neutral lipid analysis was Non-Polar Lipid Mixture-B (Matreya, Inc., Pleasant Gap, Pennsylvania), which contained 20.0% each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol and a total lipid concentration of 25.0 mg/mL. The standard was used to represent steryl esters, methyl esters, triacylglycerols, free fatty acids, and sterols, respectively, in the samples. The standard (1.00 mL) was placed in a 25 mL volumetric flask and diluted with chloroform-methanol (2:1) to prepare a standard solution containing 0.200 μg/μL for each of the components. TLC analysis was performed on Whatman high performance LHPKDF silica gel plates, 10 × 20 cm, containing 19 scored lanes and a preadsorbent spotting area. Plates were precleaned by development to the top with dichloromethane-methanol (1:1). The standards (2.00, 4.00, 8.00, and 16.00 μL) and reconstituted samples (2.00, 4.00, and 8.00 μL) were applied to the preadsorbent zone in individual lanes with a 10-μL Drummond (Broomall, Pennsylvania) digital microdispenser. Plates were developed with petroleum ether-diethyl ether-glacial acetic acid (80:20:1) mobile phase when analyzing methyl ester, triacylglycerol, free fatty acid and sterol content in the samples, and with hexane-petroleum ether-diethyl ether-glacial acetic acid (50:25:5:1) when analyzing steryl esters. All plates were developed for a distance of 9 cm in a rectangular Camag (Wilmington, North Carolina) TLC twin-trough chamber containing about 50 mL of the mobile phase and a saturation pad (Anal Tech, Newark, Delaware). Developed plates were blown dry in a fume hood with air for 5 min, sprayed with 5% ethanolic phosphomolybdic acid (PMA) solution, and heated at 115°C until blue bands appeared on a yellow background.

The standard for polar lipid analysis, Polar Lipid Mix (Matreya, Inc.), contained 25.0% each of cholesterol (sterol), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidulcholine (LPC) and a total lipid concentration of 25.0 mg/mL. The standard (1.00 mL) was placed in a 25 mL volumetric flask and diluted with chloroform to prepare a standard solution of 0.250 μg/μL for each component. TLC analysis was done on the same plates and with the same sample and standard volumes as described for the neutral lipids. Plates were developed with chloroform-methanol-water (65:25:4) mobile phase, dried for 5 min under cool air in a fume hood, sprayed with a 10% cupric sulfate solution, and then heated for 10 min at 140°C to form brown bands on a white background.

Quantitative densitometric analysis was done with a Camag TLC Scanner II using a tungsten light source set at 610 nm for neutral lipids and a deuterium source set at 370 nm for polar lipids. Other scanner settings were a slit width 4, slit length 4, and scanning speed of 4 mm/sec. The CATS-3 software was used to create a linear regression calibration curve relating the weights of the standard zones (0.400–3.20 μg for neutral lipids and 0.500–4.00 μg for polar lipids). Weights of neutral lipids and polar lipids in sample aliquots were automatically interpolated from the curve. If the areas of more than one aliquot of a particular sample were bracketed within the calibration curve, the weight corresponding to the sample area closest to the areas of the middle two standards was used to calculate the weight percent of the lipid. The equation used for calculation of weight percent in the whole bodies and DGGs is:

$$\% \text{ Lipid} = \frac{(w*R*\text{Dilution Factor}*100)}{\mu\text{g snail sample}}$$

w = μg interpolated from calibration curve,
R = [reconstitution volume (μL)]/[spotted volume (μL)].

The equation used for calculation of the concentration (mg/dL) of lipid in the blood samples is:

$$\text{mg/dL lipid} = \frac{(w*R)}{(V*10)}$$

V = plasma volume (μL).

Some of the tissue samples had to be diluted or concentrated to obtain a bracketed area within the calibration curve, and then an appropriate dilution factor was included in the calculations. For several samples, the largest aliquot spotted gave a zone with a scan area below that of the lowest standard. Therefore, the exact concentrations of the lipids in these samples could not be determined. In these cases, the limit of detection was calculated, and one-half of this value was used for statistical calculations of Student's t-test.

RESULTS AND DISCUSSION

The samples analyzed for neutral lipids showed zones with comparable migration to the standards at R_f values of 0.19 (cholesterol), 0.23 (oleic acid), 0.53 (trolein), and 0.67 (methyl oleate) in the petroleum ether-diethyl ether-glacial acetic (80:20:1) mobile phase. The R_f value of cholesteryl oleate sample and standard zones was 0.61 in the hexane-petroleum ether-diethyl ether-glacial acetic

Table 1

Weight percents of lipids in the whole bodies of snails maintained on the egg yolk (Y) and the lettuce (L) diets.

Lipid	L*	Y*
Free Sterols	0.0170 ± 0.0048**	0.0330 ± 0.0023
Free Fatty Acids	0.0168 ± 0.0043	0.0393 ± 0.011
Triacylglycerols	0.0221 ± 0.011**	0.203 ± 0.039
Methyl Esters	0.00224 ± 0.0016[a]	0.00635 ± 0.041
Steryl Esters	0.00287 ± 0.00070**	0.181 ± 0.025
Phosphatidylcholine	0.0104 ± 0.0011	0.0310 ± 0.0088
Phosphatidylethanolamine	0.00244 ± 0.00061	0.0136 ± 0.0041

* Snail bodies: Mean (weight %) ± standard error; n = 4 individual snails for each sample.

** Concentration significantly reduced (Student's t-test, $P < 0.05$) compared with snails on the Y diet.

[a] Two data points were below the limit of quantification. Weight % values of 0.000685 and 0.000510 were used for calculations, which were one-half the limit of quantification for the two samples.

acid (50:20:5:1) mobile phase. Samples analyzed for polar lipids showed sample and standard zones at R_f values of 0.28 (phosphatidylcholine, PC) and 0.47 (phosphatidylethanolamine, PE). The lysophosphatidylcholine (LPC) in the polar lipid standard was not detected with the cupric sulfate reagent at the concentrations applied, and zones that might have been LPC were not seen in samples.

Table 1 presents lipid percentages for whole bodies of the snails fed the L and the Y diets (n = 4 for each sample). Mean values of free sterols, free fatty acids, triacylglycerols, methyl esters, steryl esters, PC, and PE in snails fed on the Y diet were 1.9, 2.3, 9.2, 2.8, 63, 3.0, and 5.6 times higher, respectively, than the whole bodies of snails fed the L diet. The concentrations of free sterols, triacylglycerols, and steryl esters in the snails fed the Y diet were significantly higher than those fed the L diet (Student's t-test, $P < 0.05$).

Table 2 presents percentages of lipids in the DGGs of snails fed the lettuce versus yolk diets. The mean values for free sterols, free fatty acids, triacylglycerols, methyl esters, steryl esters, PC, and PE in snails fed the Y diet were 3.0, 2.2, 3.0, 6.4, 58, 1.4, and 3.4 times higher, respectively, than those on the L diet. The amount of steryl esters in the snails on the Y diet was significantly greater than that on the L diet (Student's t-test, $P < 0.05$).

Table 3 shows the lipids detected in the plasma of the snails on the L and Y diets. Each sample consisted of pooled plasma from three snails, and only one analysis was made for each diet. Only free fatty acids and PC were found in the pooled plasma samples. The values of the free fatty acids and PC were 1.9 and 3.4 times higher, respectively, in the Y diet than the L diet snails. The sample size precluded t-test analysis.

Snails maintained on the Y diet showed a gross difference in appearance compared to those on the L diet. The DGGs of snails fed the L diet were dark green-brown in color, while the DGGs of snails on the Y diet were yellow-white in color. The difference in the appearance of the DGGs in snails on the Y diet was apparent at about 1 week after the snails were placed on the hen's egg yolk diet. The appearance of the snail DGGs is an apparent

Table 2

Weight percent of lipids in the DGGs of snails maintained on the egg yolk (Y) and the lettuce fed (L) diets.

Lipid	L*	Y*
Free Sterols	0.0198 ± 0.0049	0.0593 ± 0.030
Free Fatty Acids	0.0265 ± 0.011	0.0593 ± 0.030
Triacylglycerols	0.0692 ± 0.012	0.207 ± 0.103
Methyl Esters	0.00290 ± 0.00080[a]	0.0185 ± 0.00924
Steryl Esters	0.00186 ± 0.00012[b]**	0.108 ± 0.058
Phosphatidylcholine	0.0334 ± 0.0035	0.0480 ± 0.011
Phosphatidylethanolamine	0.00731 ± 0.00089	0.0248 ± 0.0066

* Snail bodies: Mean (weight %) ± standard error; n = 4 individual snails for each sample (unless indicated otherwise).

** Concentration significantly reduced (Student's t-test, $P < 0.05$) compared with snails on the Y diet.

[a] One data point was below the limit of quantification. A weight % value of 0.00134 was used for calculations, which was one-half the limit of quantification.

[b] All of the four data points were below the limit of quantification. Weight % values of 0.00160, 0.00174, 0.00200, and 0.00212 were used for calculations, which were one-half the limit of quantification for the four samples.

Table 3

Concentration (mg/dL) of lipids in pooled plasma samples of snails maintained on the egg yolk (Y) and the lettuce fed (L) diets.

Lipid	L*	Y*
Free Fatty Acids	6.10	11.8
Phosphatidylcholine	1.92	6.57

* Snail plasma, pooled: n = 1 with plasma pooled from 3 snails.

reflection of the different diets used in the study. The shells of snails maintained on the L diet were less susceptible to cracking than those on the Y diet. Snails on the L diet yielded more hemolymph per snail, about 100 μL per snail, than the snails on the Y diet, which yielded about 50 μL per snail.

All five major classes of neutral lipids and two phospholipids (PC and PE) were found in detectable quantities in the DGGs and whole bodies of the snails maintained on both diets. The plasma of *H. trivolvis*, however, only showed quantifiable amounts of free fatty acids and PC. Park et al. (1991) reported that the free fatty acids were the major lipid in snail hemolymph of *H. trivolvis* (Colorado strain) maintained on a leaf lettuce-Tetramin diet. We also found that free fatty acid was the main lipid fraction in the plasma of this snail maintained on both diets.

The previous study on *H. trivolvis* (Colorado strain) maintained on a lettuce-Tetramin diet (Park et al., 1991) showed that triacylglyerols were at the highest concentration in snail DGG, followed by free sterols, free fatty acids, and steryl esters; this finding is in accord with our results on *H. trivolvis* maintained on the Y and L diets. The average triacylglycerol content in snail DGG on the L diet, 0.0221%, was considerably less than that reported by Park et al. (1991), which was 0.69% for snails on a lettuce-Tetramin diet. These differences probably reflect dietary differences in the two studies.

In conclusion, the results of this study show that the Colorado strain of *H. trivolvis* is a useful model to study the effects of a high lipid diet on hyperlipidemia and hyperlipemia in a pulmonate snail.

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